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Differential Phosphorylation of a Regulatory Subunit of Protein Kinase CK2 by Target of Rapamycin Complex 1 Signaling and the Cdc-like Kinase Kns1*[S]

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Background: Protein kinase CK2 participates in many regulated processes but the regulation of CK2 is enigmatic.
Results: A regulatory subunit of CK2 is phosphorylated under multiple stress conditions by the target of rapamycin complex 1 (TORC1) effector kinase Kns1.
Conclusion: CK2 is a downstream target of TORC1 signaling.
Significance: TOR activity via Kns1 may regulate CK2 function by affecting the interaction of the CK2 holoenzyme with its substrates.

Transcriptional regulation of ribosome and tRNA synthesis plays a central role in determining protein synthetic capacity and is tightly controlled in response to nutrient availability and cellular stress. In *Saccharomyces cerevisiae*, the regulation of ribosome and tRNA synthesis was recently shown to involve the Cdc-like kinase Kns1 and the GSK-3 kinase Mck1. In this study, we explored additional roles for these conserved kinases in processes connected to the target of rapamycin complex 1 (TORC1). We conducted a synthetic chemical-genetic screen in a *kns1*Δ *mck1*Δ strain and identified many novel rapamycin-hypersensitive genes. Gene ontology analysis showed enrichment for TORC1-regulated processes (vesicle-mediated transport, autophagy, and regulation of cell size) and identified new connections to protein complexes including the protein kinase CK2. CK2 is considered to be a constitutively active kinase and in budding yeast, the holoenzyme comprises two regulatory subunits, Ckb1 and Ckb2, and two catalytic subunits, Cka1 and Cka2. We show that Ckb1 is differentially phosphorylated *in vivo* and that Kns1 mediates this phosphorylation when nutrients are limiting and under all tested stress conditions. We determined that the phosphorylation of Ckb1 does not detectably affect the stability of the CK2 holoenzyme but correlates with the reduced occupancy of Ckb1 on tRNA genes after rapamycin treatment. Thus, the differential occupancy of tRNA genes by CK2 is likely to modulate its activation of RNA polymerase III transcription. Our data suggest that TORC1, via its effector kinase Kns1, may regulate the association of CK2 with some of its substrates by phosphorylating Ckb1.

The regulation of energy utilization in response to changing intracellular and extracellular conditions is key for survival. The target of rapamycin (TOR),[3] a protein kinase of the phosphoinositide 3-kinase family, plays a central role in signaling nutrient availability, stress conditions, and cellular energy levels to downstream anabolic and catabolic pathways to promote cell growth and prevent cell death (1, 2). As a component of TOR complex 1 (TORC1), TOR kinase activity balances the level of protein synthesis and the need for protein synthetic capacity (*i.e.* ribosome biogenesis) with cellular demand (3). Ribosome biogenesis is an energetically costly process that accounts for over 80% of the transcriptional activity in growing cells and involves the coordinated action of all three nuclear RNA polymerases (4). The regulation of ribosome biogenesis is therefore a central hub for cellular energy management that is critical for limiting growth in unfavorable conditions.

The loss of transcriptional regulation of ribosome and tRNA synthesis is highly correlated with multiple types of cancers, especially those involving tumor-suppressor genes (*e.g.* p53, Rb proteins) and oncogenes (*e.g.* c-*Myc*) that directly target transcription by RNA polymerases (pols) I and III (5–8). These correlations are not likely to be coincidental. Indeed, studies have shown that high levels of transcription by pols I and III are in fact required for cell transformation and tumorigenesis by c-*Myc* (9, 10). These studies suggest new opportunities for the development of anti-cancer therapeutics that act by "normalizing" pol I or pol III transcription.

We recently reported a role for the Cdc-like kinase, Kns1, and the GSK-3 family kinase, Mck1, in the regulation of ribosome and tRNA synthesis downstream of TORC1 (11). Focusing on the pol III system, these two kinases were shown to work in tandem to phosphorylate the TFIIF-related Rpc53 subunit of the polymerase under multiple repressing conditions and to

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[3] The abbreviations used are: TORC1, target of rapamycin complex 1; SGA, synthetic genetic array; MMS, methyl methanesulfonate; IP, immunoprecipitation; pre-tRNA, precursor tRNA; WCE, whole cell extract; TBP, TATA box-binding protein.

contribute to Maf1-mediated repression of pol III transcription (3). The data suggested a novel regulatory mechanism in which TORC1 signaling to the polymerase may interrupt the conserved process of facilitated polymerase recycling to enable repression by Maf1 (3, 11).

Protein kinase CK2 is a constitutive pro-growth serine/threonine kinase involved in the regulation of cell cycle progression, cell proliferation, transcription, and other aspects of cellular life (12, 13). Structurally, CK2 is a heterotetramer comprising a dimer of regulatory β subunits and two catalytic α subunits that associate with the α dimer (14, 15). In yeast, the catalytic subunits, α and α', are encoded by *CKA1* and *CKA2*, whereas the β and β' subunits are encoded by *CKB1* and *CKB2* (reviewed in Ref. 16). CK2 activity is essential for viability in yeast (16) and in higher eukaryotes (17, 18) and is known to regulate pol III transcription in these systems by phosphorylating one or more subunits of the initiator factor TFIIIB (19–22). How this regulation is achieved is not well defined, nor is it clear how CK2 activity or function is controlled in this context. Indeed, the regulation of CK2 is enigmatic; the kinase is not known to bind regulatory ligands and its function is not strongly connected to signal transduction pathways. CK2 function is implicated in multiple cancers and the elevated CK2 protein level and activity is thought to create a favorable environment for the development of tumors (12, 23). To our knowledge, the only signaling pathway known to regulate CK2 function involves epidermal growth factor receptor-mediated activation of ERK2, which phosphorylates and stimulates CK2α activity toward α-catenin leading to transcriptional activation of β-catenin and tumor cell invasion (24). A similar level of understanding for CK2 regulation of other processes awaits further study (12).

The role of Kns1 and Mck1 in the regulation of ribosome and tRNA synthesis downstream of TORC1 led us to hypothesize that these kinases may control other TORC1-regulated outputs. To explore this possibility, we used a synthetic chemical-genetic approach to identify new rapamycin-hypersensitive mutants connected to *KNS1* and *MCK1*. These mutants included one of the β regulatory subunits of CK2, Ckb1, which we show is phosphorylated by Kns1 under multiple conditions including TORC1 inhibition and the response to alkylating DNA damage. We show that phosphorylation of Ckb1 does not affect CK2 holoenzyme stability but correlates with decreased Ckb1 occupancy of tRNA genes after rapamycin treatment. Together our data suggest a link between TORC1 activity, Kns1 phosphorylation of Ckb1, and CK2 regulation of pol III transcription.

EXPERIMENTAL PROCEDURES

Yeast Strains and Genetic Methods—Query strains were derived from Y7092 (25) by deleting *MCK1* (*mck1*Δ::*natR*), *KNS1* (*kns1*Δ::*hphR*), or both genes (*mck1*Δ::*natR kns1*Δ::*hphR*). The double mutant query strain was screened using a RoToR HDA robot (Singer Instrument Co., UK) and standard synthetic genetic array (SGA) methods against an array of 4292 strains representing a healthy subset (*i.e.* minimal growth defects) of the viable yeast gene-deletion collection (25). The resulting triple mutant haploid colonies arrayed at a density of 384 colonies per plate were pinned into 384-well microtiter plates containing 80 μl of sterile water/well and mixed. Liquid to solid transfers were then performed onto SGA triple selection medium (with G418, nourseothricin and hygromycin) and then onto the same medium containing 10 nM rapamycin. Colonies were grown at 30 °C for 2–5 days. Digital images of the plates were obtained using an SPImager (S&P Robotics, Toronto CA) and the pixel sizes of the colonies were determined using Balony software (26). For each of the 14 array plates, the triple mutants were rank ordered by the ratio of colony sizes in the presence *versus* the absence of rapamycin. Sensitivity analysis revealed that 80% of the known rapamycin-sensitive gene deletions (27) that were confirmed under our assay conditions ranked in the 36 top scoring strains on each plate. Analysis of these colonies by 10-fold serial dilution spot assays on SGA triple selection medium ($\pm$10 nM rapamycin) validated 91 "new" rapamycin-sensitive gene deletions. These genes, along with the 129 known rapamycin-sensitive genes that were validated by spot assays, were used for gene ontology analysis (GO TermFinder) (27) and for exploring genetic and physical interactions (GeneMANIA) (28).

Strains used for Phos-tag gel analysis, immunoprecipitations, and glycerol gradients were derived from BY4742. Genes were epitope tagged using standard techniques. HA-tagged CK2 subunits were selected with clonNat (nourseothricin), whereas myc-tagged alleles were selected with G418. Strains for Northern analysis were derived from W303 and contained wild-type or phosphosite mutant *BDP1-HA*::*kanR* alleles integrated by homologous recombination. These strains were further modified by chromosomal deletion of *MAF1* and *CKB1* and transformation with plasmids containing tagged alleles of these genes (*maf1*Δ::*natR* pRS314 *MAF1-myc ckb1*Δ::*hphR* pRS313 *CKB1-HA*::*natR*).

Generation of Ckb1 Phosphosite Mutants—CKB1 was PCR-amplified from a chromosomally HA-tagged allele using primers to engineer SpeI restriction enzyme sites 650 base pairs upstream of the coding sequence and 250 base pairs downstream of the resistance marker to retain the C-terminal HA epitope tag. PCR products were restriction enzyme digested and cloned into pRS313. *CKB1* phosphosite mutants were generated using a QuikChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer's instructions. The starting wild-type clone and the mutants were verified by DNA sequencing. Phosphosite mutants were used on plasmids or re-introduced into *Saccharomyces cerevisiae* by restriction enzyme digestion followed by homologous recombination and drug selection with clonNat.

Yeast Growth and Drug Treatment—Strains were grown in YPD or synthetic complete media as required at 30 °C to A_{600} 0.4–0.5 and treated with drug vehicle (dimethyl sulfoxide), rapamycin (0.2 μg/ml), chlorpromazine (250 mM), or methyl methanesulfonate (0.08% w/v) for 1 h, or with tunicamycin (2.5 μg/ml, 3 h) or cycloheximide (200 μg/ml, 0–6 h). For phosphoprotein analysis, cells were TCA precipitated, pelleted by centrifugation at room temperature, and stored at −80 °C until processing (11).

Western Blotting, Immunoprecipitations, and Glycerol Gradient Centrifugation—Whole cell extracts (WCEs) for Phos-tag gel analysis were prepared by glass-bead breakage into urea

buffer (50 mM Tris-HCl, pH 7.5, 6 M urea, 1% SDS plus EDTA-free protease inhibitor, Roche Diagnostics). WCEs for immunoprecipitations and glycerol gradients were prepared similarly in KBC100 buffer (20 mM HEPES-KOH, pH 7.9, 100 mM KCl, 20% glycerol, 2 mM DTT, 0. 2 mM EDTA, plus phosphatase and protease inhibitors). WCEs were cleared by centrifugation (5 min at 15,000 × *g* at 4 °C) (11, 29). Protein concentration was quantified using Pierce BCA protein assay kit (Thermo Scientific). Cleared WCEs (50 μg) were separated by Phos-tag SDS-PAGE (Phos-tag from NARD Institute, used at 25 μM final concentration). For immunoprecipitations, WCEs (300 μg) were incubated with monoclonal antibodies (anti-HA 12CA5, anti-myc 9E10, Roche Applied Sciences) or antisera (anti-HA Y-11, Santa Cruz Biotechnology, or preimmune or TBP antisera) at 4 °C for 2 h with end-over-end rotation. Protein G-Sepharose 4 Fast Flow beads (30 μl, 50% slurry, Amersham Biosciences) were added for an additional 2-h incubation at 4 °C. Beads were washed 3 times in KBC100 and bound proteins were eluted into 1× Laemmli buffer for SDS-PAGE and transfer to nitrocellulose membranes. Membranes were probed with the indicated primary antibodies and Infrared IRDye® secondary antibodies (Li-Cor). Signals were detected with an Odyssey infrared imaging system and quantified using Image Studio Software (Li-Cor). CK2 subunit abundance was reduced in saturated cultures (~5-fold for catalytic subunits and ~2-fold for Ckb1) relative to log phase. To account for these changes in co-immunoprecipitations, a "normalization factor" was calculated by dividing the myc/HA ratio for log cultures by the myc/HA ratio of high cell density cultures. This normalization factor was applied to the calculated IP ratio for log cultures. The normalized value was then compared with the raw co-IP ratio of high cell density cultures to query the association of CK2 subunits in both conditions. The normalization of methyl methanesulfonate (MMS) treatment experiments did not alter the IP ratios because changes in subunit composition were minimal under these conditions. Glycerol gradients (5–20%) were carried out as described previously (30). Protein stability was examined by addition of cycloheximide (200 μg/ml) to early-log phase (A_{600} ~ 0.4 – 0.5) cultures in YPD. Aliquots (20 ml) were harvested at various times for extract preparation and Western blotting by standard SDS-PAGE as described above.

Chromatin Immunoprecipitation—Chromatin samples were prepared according to established protocols (31, 32). Briefly, cultures with or without drug treatment were formaldehyde-fixed (1% for 20 min), pelleted, washed twice with TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and once with FA lysis buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS). Cells were lysed by vortexing with glass beads in FA lysis buffer and the DNA was sonicated to an average fragment size of 500 bp. Chromatin extracts were thawed on ice, adjusted to 275 mM NaCl, and pre-cleared by incubation with Protein G-Sepharose 4 Fast Flow beads for 1 h. Cleared extracts were immunoprecipitated overnight at 4 °C with anti-HA antibody or no antibody. Beads were washed four times with FA lysis buffer and DNA cross-links were reversed following digestion with Pronase (20 μl, 20 mg/ml, Roche Applied Science, 2 h at 42 °C) by incubation for 4 h at 65 °C. DNA was purified using a QIAquick PCR purification kit (Qiagen). Quantitative Real-time PCR was performed using a LightCycler 480 (Roche) with SYBR Green I master mix and oligonucleotide primers as described previously (29). Relative occupancy was determined using the $\Delta\Delta C_t$ method (33) comparing immunoprecipitated and input samples and normalizing these values to an intergenic region on chromosome V (34). Errors were calculated by applying the root mean square deviation to the occupancy values (33).

Northern Analysis—RNA was prepared by hot phenol extraction, separated on denaturing urea/polyacrylamide gels, and transferred to Nytran-N membranes for hybridization to oligonucleotide probes as described previously (11, 35).

In Vitro Kinase Assay—The coding regions of wild-type or S111A Ckb1 were PCR amplified using oligos to engineer a NcoI restriction enzyme site N-terminal to the ATG start site and a XhoI site at the C terminus. These products were digested and ligated into pET21-d to introduce a C-terminal histidine tag and expressed in Rosetta2 (DE3) cells. Recombinant Ckb1-His_6 and His_6-Kns1 proteins were purified and used for *in vitro* kinase assays as described (11).

RESULTS

A Chemical-Genetic Screen for Rapamycin Hypersensitivity—Sensitivity to rapamycin is a multigenic trait with several hundred single gene deletions known to alter cell growth in the presence of the drug in *S. cerevisiae* (27). These chemical-genetic interactions identify genes that are likely to function in TORC1-regulated processes. In previous work, we identified Kns1 and Mck1 as downstream effectors of TORC1 in the regulation of ribosome and tRNA synthesis (11). We reasoned that deletion of one or both kinases may lead to rapamycin hypersensitivity. Indeed, we found that deletion of *MCK1* but not *KNS1* increased rapamycin sensitivity compared with a wild-type strain, whereas deletion of both *KNS1* and *MCK1* markedly reduced growth on rapamycin-containing media (Fig. 1*A*). Thus, deletion of *MCK1* uncovered the sensitivity of the *KNS1* deletion to rapamycin. We chose to extend this approach in an effort to identify new genes whose sensitivity to TORC1 inhibition might be revealed by deletions of both *MCK1* and *KNS1*. A *kns1*Δ *mck1*Δ query strain was constructed and crossed to an array of viable single gene deletions using SGA methods (25). The resulting triple mutant haploid strains were then assayed for their ability to grow on solid medium in the presence or absence of rapamycin. Digital images of the plates were used to calculate colony sizes and determine the relative drug sensitivity of the triple mutants.

Using this approach we identified many gene deletion strains that are known to be rapamycin-sensitive (supplemental Table S1). In addition, we recovered a set of gene deletion strains that had not previously been associated with this phenotype. These triple mutant strains were further examined by performing serial dilution spot assays on medium with and without the drug. Visual scoring of growth relative to the parental double mutant strain verified 91 new gene deletions that confer rapamycin hypersensitivity in the *kns1*Δ *mck1*Δ background (supplemental Table S1). Two additional miniarray screens were then performed in which the 91 single gene deletion strains

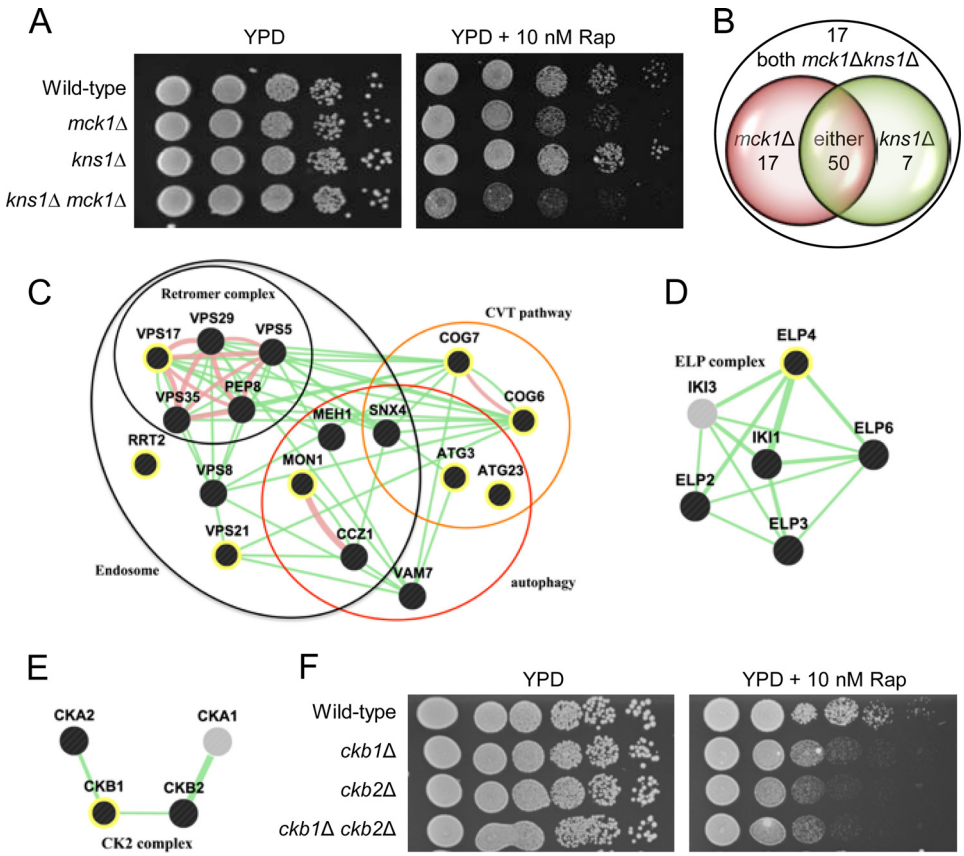

FIGURE 1. **Deletion of *KNS1* and *MCK1* sensitizes strains to rapamycin treatment.** *A,* 10-fold serial dilutions starting at optical density 1.0 were spotted on YPD media in the absence or presence of 10 nM rapamycin (*Rap*). Plates were incubated at 30 °C for 2 or 5 days, respectively. *B*, venn diagram of the 91 newly identified rapamycin-sensitive genes. The overlapping area represents gene deletions that are rapamycin sensitive in *kns1*Δ and *mck1*Δ strains. Strains labeled "both" represent gene deletions that are only rapamycin-sensitive upon deletion of both kinases. *C*, Gene Ontology categories involved in vesicle-mediated transport are graphically represented using GeneMANIA (28). Genetic interactions are depicted in *light green* based on the Costanzo-Boone 2010 positive and negative interaction intermediate dataset (61); physical interactions are shown in *light brown*, based on the Collins-Krogan 2007 dataset (62). The nodes corresponding to new rapamycin-sensitive genes identified in this study are highlighted in *yellow*. *CVT*, cytoplasm-to-vacuole pathway. *D*, the six subunits of the Elongator complex are represented as above. IKI3, represented by the *gray node*, was not identified in this study. *E*, graphical representation of the four subunits of protein kinase CK2. CKA1 (*gray node*) was not identified in this study, whereas CKB1 (*yellow node*) represents a newly identified rapamycin-sensitive gene-deletion. *F,* 10-fold serial dilutions of the indicated strains were spotted on YPD media in the absence or presence of 10 nM rapamycin and incubated as described in *panel A*.

were crossed to strains deleted for either *KNS1* or *MCK1* to quantify the contribution of each kinase to the original triple mutant phenotype. Genes were assigned to one of four groups (Fig. 1*B* and supplemental Table S1). These groups represent gene deletions that require both kinases to be deleted for rapamycin hypersensitivity (annotated "both *kns1*Δ *mck1*Δ" in Fig. 1*B*), gene deletions that are sensitive to the absence of only one kinase, and gene deletions that are sensitive to the absence of either kinase (overlap, annotated "either" in Fig. 1*B*).

Gene ontology analysis identified numerous biological processes and discrete protein complexes connected to TORC1 (Table 1 and supplemental Table S2). Highly enriched processes correspond to vesicle-mediated transport, autophagy, and the regulation of cell size and cell polarity. Vesicle-mediated transport, in particular, distills highly related processes including Golgi vesicle transport, retrograde transport, and endosome-to-Golgi transport, and indicates a high connectivity of new and known rapamycin-sensitive targets (Fig. 1*C*). Autophagy was also enriched, along with protein localization to the vacuole, an important step in the autophagic response (36). In addition to biological processes, specific protein complexes were enriched in the screen. One example is the Elongator complex (*ELP*, Fig. 1*D*) required for the modification of uridine at the wobble position in certain tRNAs (37). Five of the six members of the Elongator complex were recovered, including *ELP4*, whose single gene deletion was not previously annotated as rapamycin-sensitive. Another complex identified in the screen was the protein kinase CK2 (Fig. 1*E*). In yeast, deletion of *CKA2* or *CKB2* results in rapamycin sensitivity (27), and these deletions were also rapamycin-sensitive in the *kns1*Δ *mck1*Δ background. In addition, deletion of *CKB1* in this context uncovered a rapamycin-sensitive phenotype. Subsequently, we found that *ckb1*Δ, like *ckb2*Δ, is rapamycin-sensitive as a single gene deletion (Fig. 1*F*).

Ckb1 Is Phosphoregulated Under Stress by Kns1—Rapamycin sensitivity of the CK2 subunit gene deletions suggested that CK2 may be involved in TORC1 signaling, potentially as a direct or indirect target of TORC1 phosphoregulation. Little is known about the phosphorylation of CK2 subunits *in vivo* in *S. cerevisiae*, although evidence of *in vivo* phosphorylation of human CK2 subunits has been reported (38, 39). We tested whether CK2 may be differentially phosphorylated after rapa-

TABLE 1

Gene ontology processes enriched in the screen

The gene ontology bioprocess term enrichment was conducted using goTermFinder (see supplemental Table S2 for the full dataset). New rapamycin-sensitive gene-deletions identified in this work are noted in bold.

GOID	GO_term	Cluster frequency	Background frequency	*p* value	Gene(s) annotated to the term (new in bold)
16192	Vesicle-mediated transport	38 out of 220 genes, 17.3%	348 out of 7167 background genes, 4.9%	3.47E-09	CCZ1, ARL1, **RRT2**, **ARF1**, VPS41, SWF1, SWA2, SEM1, **VPS74**, **PKH1**, BST1, **COG7**, ERV14, **MON1**, VAM7, GOS1, VPS29, SYS1, SNX4, PEP8, VPS35, RAV1, LST4, CHS5, **FKS1**, **ROM2**, ELO3, YPT7, VPS9, **COG6**, TPM1, **SLG1**, VPS5, **VPS21**, **VPS17**, SNX3, ARL3, SRO7
6914	Autophagy	18 out of 220 genes, 8.2%	105 out of 7167 background genes, 1.5%	2.03E-06	CCZ1, PTC6, **ARF1**, VPS41, VAC8, **MON1**, VAM7, GTR2, SLT2, SNX4, BCK1, AIM26, MEH1, **ATG23**, YPT7, **ATG3**, PHO80, **SLG1**
2097	tRNA wobble base modification	10 out of 220 genes, 4.5%	27 out of 7167 background genes, 0.4%	2.99E-06	ATS1, NCS6, ELP2, UBA4, URM1, IKI3, ELP6, NCS2, ELP3, **ELP4**
8361	Regulation of cell size	10 out of 220 genes, 4.5%	32 out of 7167 background genes, 0.4%	2.00E-05	**PHO5**, RPA14, **GPA2**, **KAP122**, SLT2, PIH1, **SKN7**, **SWE1**, **FKS1**, WHI3
30010	Establishment of cell polarity	15 out of 220 genes, 6.8%	84 out of 7167 background genes, 1.2%	2.63E-05	SWF1, ERV14, BCK1, **ELM1**, DYN1, **LDB18**, **BUD6**, CHS5, **ROM2**, ZDS1, JNM1, TPM1, BNI1, **SLG1**, SRO7
10468	Regulation of gene expression	44 out of 220 genes, 20.0%	682 out of 7167 background genes, 9.5%	0.00111	**SWD1**, **RPS9B**, ISW1, **SGF29**, SSD1, STP1, **PKH1**, **CKB1**, DST1, **GCN1**, GTR2, ELP2, **SKN7**, **GZF3**, SET2, **CBF1**, TIF1, **MLP1**, **BRE2**, **STM1**, YKE2, CHS5, IKI3, CDC73, GTR1, **RCO1**, **YKU80**, ZDS1, GAS1, ELP6, **PUB1**, WHI3, YNL296W, CSE2, PHO80, **HTZ1**, **MSH2**, CKB2, CKA2, **LEO1**, ELP3, **ELP4**, **EAF3**, ROX1

mycin treatment by analyzing the migration of each subunit on denaturing Phos-tag gels, which retard the migration of phosphorylated species (11, 40). No indication of differential phosphorylation was obtained for Cka1, Cka2, or Ckb2; each of these proteins appeared as a single band whose migration was unaffected by rapamycin treatment (Fig. 2*A*). In contrast, two major bands were identified for Ckb1 and the abundance of these bands changed in response to rapamycin (Fig. 2*A*); the slow-migrating band increased in abundance upon inhibition of TORC1 consistent with increased phosphorylation of Ckb1 under these conditions. This was confirmed by additional experiments as described below.

Transcription by RNA pol III is repressed during nutrient limitation and under multiple environmental and cellular stress conditions. These conditions lead to changes in the phosphorylation of RNA pol III transcription components (*e.g.* Rpc53) as well as the transcriptional repressor Maf1 (3). From these studies and the known role of CK2 in promoting RNA pol III transcription in yeast (20) we hypothesized that Ckb1 phosphorylation would not be limited to rapamycin-mediated inhibition of TORC1. Indeed, we found Ckb1 to be differentially phosphorylated under a range of conditions that repress RNA pol III transcription including DNA damage, cell wall stress, ER stress, and growth to high cell density (Fig. 2*B*, *top panel*). Under the conditions employed, the conversion of Ckb1 from the fast to the slow migrating form in the Phos-tag gel system increased from 3% in early log phase cells to >40% in nutrient-limited, saturated cultures and after tunicamycin treatment (Fig. 2*B*, *top panel*).

We queried the available phosphoproteomic data for kinase and phosphatase deletion mutants in yeast that altered Ckb1 phosphorylation (41). Of the non-essential kinases reported to decrease Ckb1 phosphorylation, deletion of Kns1 significantly changed the abundance of a peptide containing a single phosphorylated residue, Ser-111. Importantly, this serine residue occurs within a consensus motif for Kns1 (Fig. 2*C*) (11). We previously identified this motif in Rpc53 and demonstrated its Kns1-dependent phosphorylation under repressing conditions *in vivo* as well as its phosphorylation by recombinant Kns1 *in vitro* (11). Based on this information, we next tested the role of Kns1 in Ckb1 phosphorylation by exposing a *kns1*Δ strain to the same repressing conditions as described above (Fig. 2*B*, *middle panel*). Notably, the slow-migrating Ckb1 band was barely detectable in every condition. A similar result was obtained for a serine to alanine mutant of Ckb1 (S111A) that disrupts the Kns1 phosphorylation site (Fig. 2*B*, *bottom panel*). We then tested the ability of recombinant Kns1 to phosphorylate Ckb1 *in vitro*. Kns1 phosphorylated wild-type Ckb1, whereas its activity toward the S111A phosphosite mutant was greatly reduced (Fig. 2*D*). Furthermore, a kinase-dead Kns1 mutant was unable to phosphorylate Ckb1. Together these data show that (i) Ckb1 is differentially phosphorylated under stress, (ii) Ser-111 is the major *in vivo* phosphosite in Ckb1, and (iii) the phosphorylation of Ckb1 is mediated directly by Kns1 under all tested repressing conditions. These results suggest that phosphorylation of Ckb1 may regulate the function of CK2.

CK2 Subunits Do Not Dissociate in Response to Limiting Nutrients or Cellular Stress—Transcription by RNA pol III is high during logarithmic growth and repressed in settings of nutrient limitation or cell stress, including DNA damage (3, 20). CK2 contributes to the efficiency of RNA pol III transcription (19) via the phosphorylation of one or more subunits of the initiator factor TFIIIB (42). Furthermore, in strains expressing GST-tagged CK2 subunits, the α′ catalytic subunit was reported to dissociate from the CK2-TBP complex in response to alkylating DNA damage (20). To examine the association of CK2 with TBP, we carried out TBP immunoprecipitations in extracts of chromosomally tagged *CKB1-HA CKA1-myc* strains and tested for co-immunoprecipitation of CK2 subunits (Fig. 3*A*). Whereas TBP was specifically and efficiently immunoprecipitated as expected, the Ckb1-HA signal was less than 0.3% of input and Cka1-myc was barely detectable. Thus, under our assay conditions, CK2 is not strongly associated with TBP.

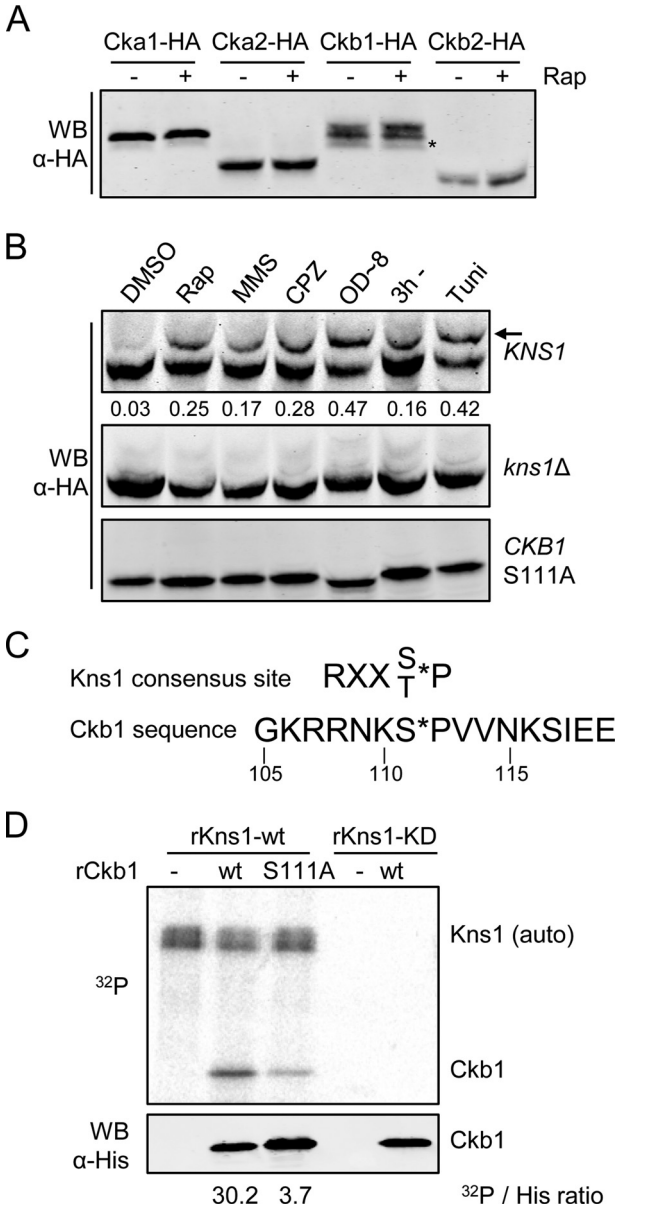

FIGURE 2. **Ckb1 is phosphorylated by Kns1 in multiple stress conditions.** *A*, extracts from control and rapamycin-treated HA-tagged strains were separated by Phos-tag SDS-PAGE and Western blots (*WB*) detected with an anti-hemagglutinin (HA) antibody (WB α-HA). The faint, fast-migrating Ckb1-HA band (*asterisk*) is a presumed degradation product that was not reproducibly detected. *B*, Phos-tag SDS-PAGE separation of Ckb1-HA in *KNS1* wild-type, *kns1*Δ, or *CKB1* S111A cell extracts. Early log phase cultures were treated with dimethyl sulfoxide (*DMSO*), rapamycin (*Rap*), MMS, or chlorpromazine for 1 h or tunicamycin (*Tuni*) for 3 h. Control cells for tunicamycin were grown for an additional 3 h (*3h*−) in the absence of drug. Extracts from high cell density ($A_{600} \sim 8$) cultures are also shown. The *arrow* indicates phosphorylated Ckb1. The fraction of total Kns1 that is phosphorylated is indicated *under* each lane. *C*, Ckb1 Ser-111 conforms to the Kns1 consensus site R*XX*(S/T)P. *Asterisk* indicates the phosphorylated residue. *D*, *in vitro* phosphorylation of recombinant wild-type and S111A Ckb1 proteins by recombinant wild-type or kinase-dead (*KD*) Kns1. [γ-^{32}P]ATP-labeled products were resolved by standard SDS-PAGE and show Kns1 autophosphorylation (*auto*) and labeling of Ckb1. Radioactivity incorporated into Ckb1 was normalized to the Ckb1-His_6 signal detected by anti-His antibody (WB α-His) and reported as a ^{32}P/His ratio in arbitrary units.

Given the high stoichiometry of Ckb1 phosphorylation under repressing conditions (Fig. 2*B*), we next tested whether Ckb1 phosphorylation would lead to the dissociation of CK2 catalytic and regulatory subunits after cell stress. We used the ability of the catalytic subunits to co-immunoprecipitate with Ckb1 as a measure of CK2 holoenzyme stability. Immunoprecipitations were performed using extracts from logarithmically growing or high cell density cultures where we observe maximal Ckb1-HA phosphorylation (Fig. 2*B*). Although the abundance of CK2 subunits is reduced in saturated cultures, the ratio of the myc and HA signals was the same in immunoprecipitates of HA-tagged Ckb1 from the log and high cell density extracts (Fig. 3*C*, *left panel*). Similarly, reciprocal immunoprecipitations of myc-tagged Cka1 did not reveal any change in the HA/myc ratio (Fig. 3*D*, *left panel*). Thus, similar proportions of Cka1 and Ckb1 are associated in the two extracts. In parallel experiments where the Cka2 catalytic subunit was tagged (Cka2-myc), a small reduction in its co-immunoprecipitation ratio was observed in the Ckb1-HA pull-down (Fig. 3*C*, *right panel*). However, no difference was seen in reciprocal immunoprecipitations (Fig. 3*D*, *right panel*). We considered that the absence of a differential in the association of Ckb1 with the catalytic subunits might have resulted from the dephosphorylation of Ckb1. However, the level of phosphorylation of Ckb1 in immunoprecipitates was comparable with the input samples (Fig. 3*B*).

We also examined CK2 subunit associations by co-immunoprecipitation using extracts from control cells and cells treated with MMS (Fig. 4). Notably, a 1-h treatment with MMS induces Ckb1 phosphorylation without affecting the abundance of CK2 subunits (Fig. 2*B*). Ratios of co-immunoprecipitated CK2 subunits were not reduced after MMS treatment for either Ckb1 or Cka2 subunit pulldowns, despite the preservation of Ckb1-HA phosphorylation (Fig. 4*C*). These data indicate that CK2 catalytic and regulatory subunits do not dissociate to any significant degree under two repressing conditions, growth to saturation or exposure to alkylating DNA damage.

To further examine the stability of the CK2 holoenzyme under cellular stress, we adapted a glycerol gradient sedimentation assay previously used to analyze the association of CK2 in the CURI complex (30). WCEs from control and tunicamycin-treated cells were subjected to centrifugation and fractionated to analyze the distribution of CK2 subunits by Western blotting (Fig. 5*A*). Tunicamycin was used as a stress condition because it leads to near-maximal levels of Ckb1 phosphorylation (Fig. 2*B*) without affecting subunit abundance. In control extracts, Ckb1-HA and Cka2-myc subunits are enriched and co-sediment in fractions 13 and 15, marking the position of the holoenzyme (Fig. 5*A*, −*Tuni*). The Cka2-myc peak fraction (fraction 17) is largely devoid of Ckb1 and indicates a lower apparent molecular mass, consistent with a pool of free catalytic subunit. Fractions obtained from tunicamycin-treated extracts showed the same distributions for both Ckb1-HA and Cka2-myc, respectively (Fig. 5*A*, +*Tuni*). In addition, the distributions of these CK2 subunits were not affected following fractionation of a WCE from the *CKB1-HA S111E* mutant strain (Fig. 5*B*). These data support the view that CK2 subunits do not dissociate under stress conditions that lead to significant phosphorylation of Ckb1.

To confirm that the CK2 holoenzyme was resolved from the free subunits in these experiments, we compared the sedimentation of extracts from wild-type and *ckb2*Δ strains. In yeast, the

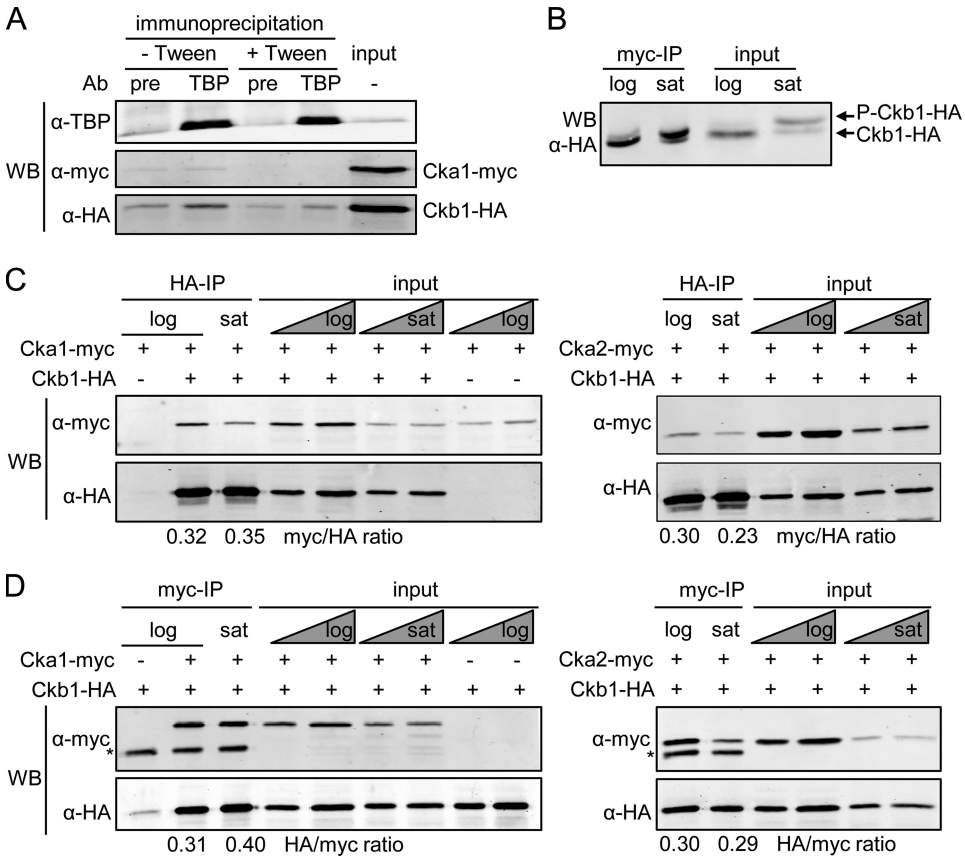

FIGURE 3. **CK2 subunit association in log and stationary phase cell extracts.** *A*, whole cell extracts of a doubly tagged CK2 strain (*CKA1-myc CKB1-HA*) were IP with either TBP antibody (*TBP*) or control preimmune sera (*pre*). Beads were washed in the presence or absence of 0.5% Tween 20 to limit nonspecific interactions. IPs and 30 μg (5%) of the input sample were separated by SDS-PAGE and detected with the indicated antibodies. *B*, Phos-tag SDS-PAGE separation of input Cka1-myc Ckb1-HA extracts and myc-IPs detected with an anti-HA antibody. *C*, IPs of tagged CK2 subunits in extracts from logarithmic (*log*) or high cell density (*sat*) cultures using a polyclonal anti-HA antibody. Untagged Ckb1 Cka1-myc extracts were used as a negative IP control. *Left*, Cka1-myc Ckb1-HA extracts; *right*, Cka2-myc Ckb1-HA extracts. Standard SDS-PAGE blots were detected with monoclonal HA and myc antibodies. The myc/HA ratios for co-immunoprecipitated CK2 subunits (indicated below the IP lanes) were normalized for subunit abundance in the extracts (see "Experimental Procedures"). *Input* lanes contain 5 and 10% of the amount used for IP. *D*, reciprocal IPs of tagged CK2 subunits from the same extracts as in *panel C* using an anti-myc antibody. Input amounts were as described in *panel C*. Untagged Cka1 Ckb1-HA extracts were used as a negative IP control. The HA/myc ratios for co-immunoprecipitated CK2 subunits (indicated *below the IP lanes*) were normalized as above. The HA signal in the negative control sample is <1% of the IP input. An *asterisk* (*) indicates the position of the IgG band in IP lanes. *WB*, Western blot.

formation of the Ckb1-Ckb2 heterodimer is required for assembly of the CK2 holoenzyme (43, 44). Thus, *ckb2*Δ extracts provide an abundant source of monomeric CK2 subunits. Extracts of logarithmically growing wild-type *CKB2* or *ckb2*Δ strains were prepared and fractionated as before. Sedimentation in these gradients (Fig. 5*C*) was reduced slightly compared with Fig. 5*A*. Ckb1-HA is distributed between fractions 13–17 and peaks in fraction 15 (Fig. 5*C*), rather than being equally distributed between fractions 13 and 15 (Fig. 5*A*). The major peaks for Cka2-myc are in fractions 17 and 19 rather than 15 and 17 (Fig. 5, *C* and *A*, respectively). Nonetheless, the peak distribution of both subunits becomes focused at fraction 17 in the *ckb2*Δ extract, confirming that this fraction contains the monomeric Cka2 and Ckb1 subunits. The total Ckb1-HA signal in *ckb2*Δ extracts was greatly decreased compared with *CKB2* wild-type extracts, suggesting rapid degradation of Ckb1 in the absence of Ckb2. This was confirmed by measuring the protein half-life in cells treated with cycloheximide (Fig. 5*D*, Ckb1 $t_{1/2} \sim 40$ min in the *ckb2*Δ strain *versus* >6 h in the wild-type strain). Together, the co-immunoprecipitation and glycerol gradient data do not support any significant dissociation of CK2 subunits in response to nutrient deprivation or cellular stress.

Reduced Association of CK2 with tRNA Gene Loci After Rapamycin Treatment—Chromatin immunoprecipitation (ChIP) has allowed extensive and accurate mapping of pol III factors and the polymerase on individual genes and over whole genomes (45–47). In addition to the transcription machinery, ChIP experiments have also provided evidence for the association of proteins other than the canonical pol III factors at pol III gene loci (48), including CK2, which has been found at pol III genes in human cells (22), *Schizosaccharomyces pombe* (31), and *S. cerevisiae* (49). Based on these data, we hypothesized that CK2 may be differentially associated with pol III genes after stress. Chromatin immunoprecipitations of the Bdp1 subunit of TFIIIB were performed in extracts from logarithmically growing cells as a reference for occupancy of pol III-transcribed genes (Fig. 6*A*). Bdp1-HA was enriched >400-fold at three different tRNA genes, relative to an intergenic region, in agreement with the high occupancy and stability of this factor at pol III genes (45). CK2 occupancy at the same genes was then analyzed before and after rapamycin treatment in a *CKB1-HA*

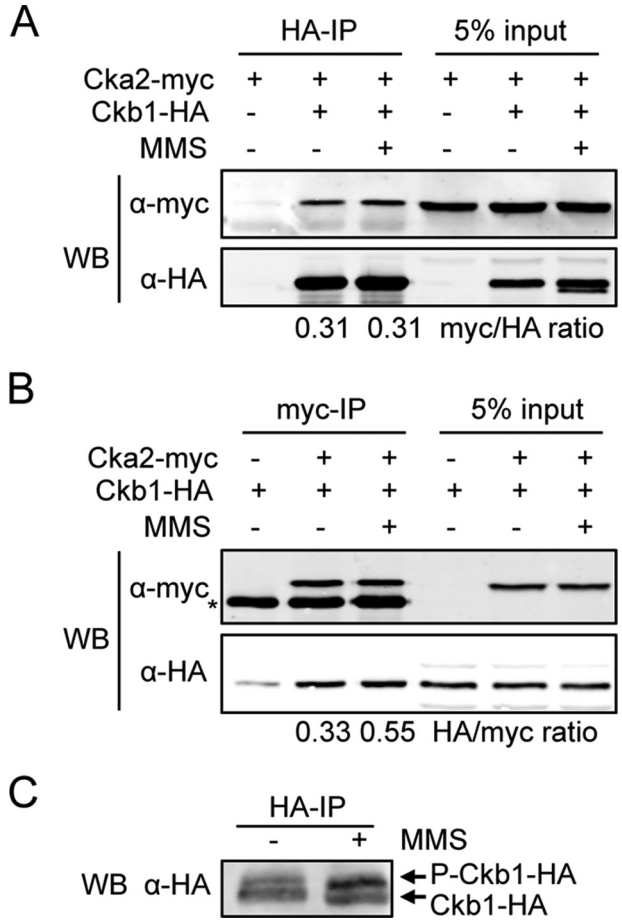

FIGURE 4. **CK2 subunit association before and after DNA damage.** IPs of tagged CK2 subunits in extracts from untreated or MMS-treated early log phase cells were detected and normalized as described in the legend to Fig. 3. *A*, IP of Ckb1-HA with a polyclonal anti-HA antibody. Cka2-myc was detected with myc antibody. An untagged Ckb1 Cka2-myc extract was used as a negative IP control. *B*, reciprocal IPs of tagged CK2 subunits were carried out using the same extracts as in *panel A*. Untagged Cka2 Ckb1-HA extract was used as a negative IP control (the HA signal is <1% of input). Calculated HA/myc co-IP ratios are indicated *below* the IP lanes. The *asterisk* (*) indicates the position of the IgG band in IP lanes. *C*, Phos-tag SDS-PAGE separation of HA-IPs was detected with HA antibody. *WB*, Western blot.

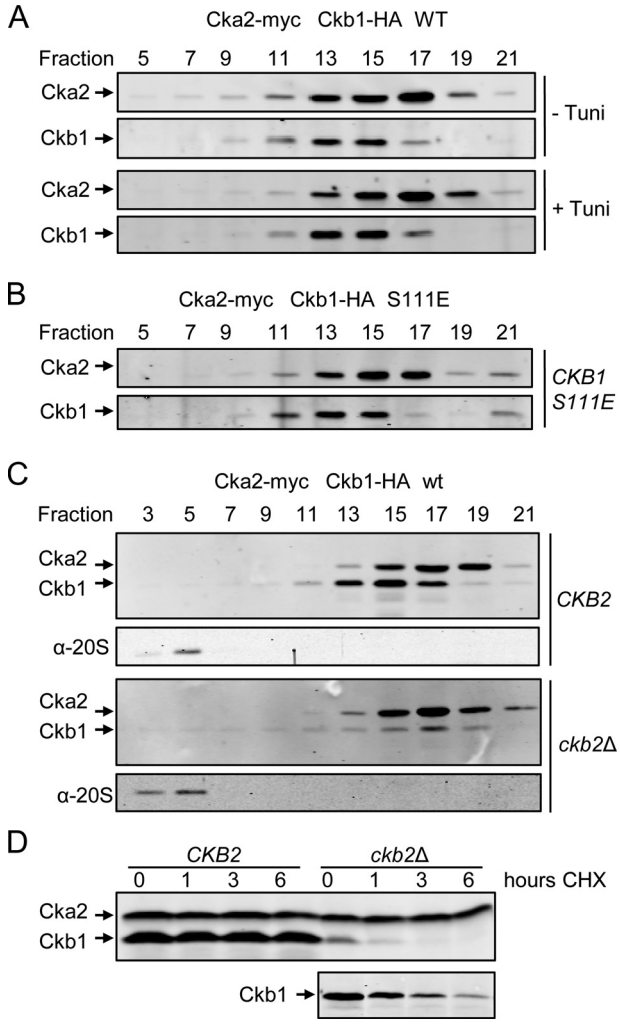

FIGURE 5. **Glycerol gradient analysis of CK2 subunit association.** Extracts from Cka2-myc Ckb1-HA-tagged strains were subjected to glycerol gradient sedimentation (5–20%). Aliquots of the indicated fractions were separated by SDS-PAGE and detected sequentially with anti-myc and anti-HA antibodies. *A*, gradients of extracts from control and tunicamycin-treated *CKA2-myc CKB1-HA* strains. *B*, gradient of a *CKA2-myc CKB1-HA S111E* phosphosite mutant strain extract. *C*, gradients of extracts from *CKB2* and *ckb2*Δ strains. Column resolution and fraction alignment was confirmed using an antibody against a subunit of the 20 S proteasome (α-*20S*). *D*, Western blot of Cka2-myc and Ckb1-HA from cycloheximide-treated *CKB2* and *ckb2*Δ strains. The *lower panel* shows an independent blot of Ckb1-HA in the *ckb2*Δ strain.

strain (Fig. 6, *B* and *C*). CK2 was enriched 2–3-fold, much lower than for Bdp1-HA, but comparable with the CK2 occupancy of tRNA genes measured in an independent study during log phase in a *CKA1-myc* strain (49). Importantly, the CK2 occupancy of the tested loci decreased significantly after rapamycin treatment, consistent with dissociation of CK2 from pol III-transcribed genes under conditions that repress their transcription. Notably, in multiple independent ChIP experiments, the dissociation of CK2 from tRNA genes was not complete suggesting that CK2 may continue to visit these genes, albeit less often, when transcription is repressed.

To determine whether the changes in CK2 occupancy that occur in response to loss of TORC1 signaling could be recapitulated or abrogated by the phosphorylation state of Ckb1, we performed ChIP experiments in strains containing S111E and S111A phosphosite mutants. The occupancy of the S111E mutant was similar to that of wild-type Ckb1 for both untreated and rapamycin-treated samples (Fig. 6*B*). Thus, the Ckb1 phosphomimetic is not sufficient to promote the dissociation of CK2 from chromatin in cells undergoing pol III transcription or enhance its dissociation after rapamycin treatment. The S111A mutant also had a similar level of occupancy to wild-type Ckb1 in logarithmically growing cells (Fig. 6*C*). The inability of Ckb1 to be phosphorylated under repressing conditions did not protect CK2 from dissociation after rapamycin treatment. Thus, changing the phosphorylation state of Ckb1 is not sufficient, in the absence of any other manipulations, to alter CK2 occupancy of pol III genes.

Analysis of Pol III Transcription in CKB1 Mutant Strains—The regulatory subunits of CK2 have been implicated in the selectivity of the kinase toward its substrates (50). Thus, the phosphorylation of Ckb1 at Ser-111 may be responsible for changes in CK2 activity against targets in the pol III system, leading to effects on transcription. To test this hypothesis, Northern blotting was used to compare pol III activity before

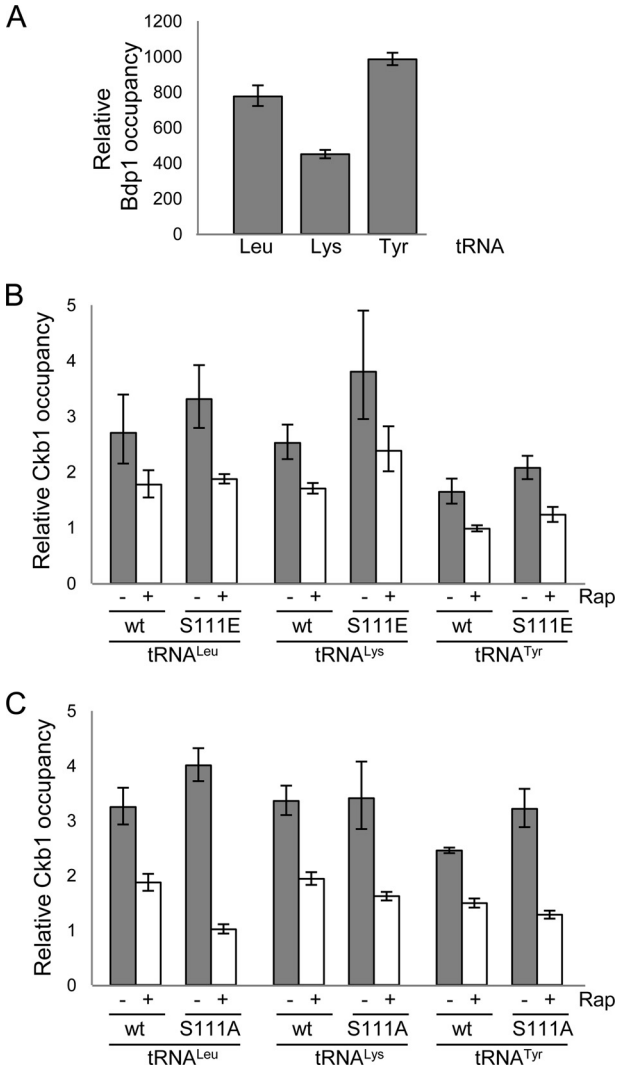

FIGURE 6. **CK2 occupancy at tRNA genes is reduced after TORC1 inhibition.** The occupancy of Bdp1 and Ckb1 was determined by chromatin immunoprecipitation at three tRNA genes in log phase cells. Occupancy values were calculated from real-time quantitative PCR values using the $\Delta\Delta C_t$ method relative to an intergenic region on chromosome V (see "Experimental Procedures"). *A*, Bdp1-HA occupancy at $tRNA^{Leu}$, $tRNA^{Lys}$, and $tRNA^{Tyr}$ loci. *B*, Ckb1 occupancy in wild-type *CKB1-HA* or *CKB1-HA S111E* mutant strains in control and rapamycin-treated cells. *C*, Ckb1 occupancy in wild-type *CKB1-HA* or *CKB1-HA S111A* mutant strains in control and rapamycin-treated cells.

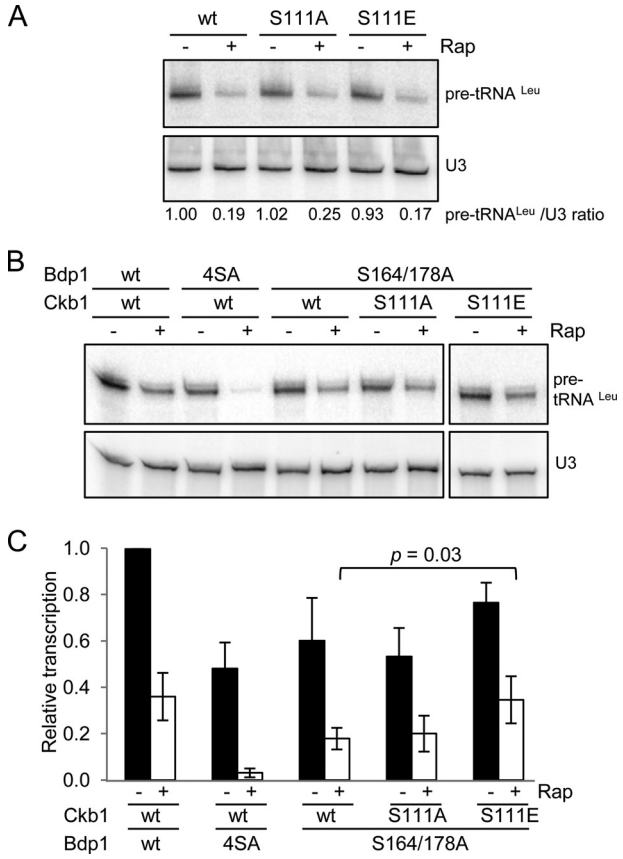

FIGURE 7. **pol III gene transcription in *CKB1-HA* phosphosite mutant strains.** Northern analysis of total RNA detected for precursor $tRNA^{Leu}$ (pre-$tRNA^{Leu}$) and control U3 snRNA. Relative transcription was calculated as the ratio of the pre-$tRNA^{Leu}$/U3 signals normalized to the value obtained for the untreated wild-type *CKB1-HA* strain. *A*, representative Northern blot of pre-$tRNA^{Leu}$ and U3 RNA from *CKB1-HA, CKB1-HA S111A*, and *CKB1-HA S111E* control and rapamycin-treated (*Rap*) cells. Quantitation of independent biological replicates is shown *below* each lane. *B*, representative Northern blot of RNA from *BDP1* wild-type, *BDP1 4SA*, and *BDP1 S164S/A178A* control and rapamycin-treated (*Rap*) cells. This particular strain background, *ckb1*Δ *maf1*Δ pRS314 *MAF1-myc*, contained pRS313 plasmid-borne *CKB1-HA* wild-type, *CKB1-HA S111A* and *CKB1-HA S111E* alleles. The S111E panel is from non-adjacent lanes on the same gel. *C*, quantitation of data in *B* derived from three independent biological replicates. *Errors* are standard deviations. Statistical significance was determined by Student's *t* test.

and after TORC1 inhibition in strains containing chromosomally tagged wild-type *CKB1-HA* or its phosphosite mutants, S111A and S111E. Using our standard reporter for tRNA synthesis (11, 35), the levels of precursor $tRNA^{Leu}$ were found to be equivalent for all three strains during log phase growth and similar levels of repression were obtained after rapamycin treatment (Fig. 7*A*). The same result was obtained for MMS and for rapamycin treatment in *ckb1*Δ strains where wild-type Ckb1-HA or the phosphosite mutants were expressed from a centromeric plasmid (51). Thus, interfering with Ckb1 phosphoregulation, by itself, is not sufficient to alter pol III transcription or its repression under stress.

The robustness of the pol III system against perturbations to regulatory modifications has been observed in several different contexts (11, 35). For example, Maf1 6SA or 7SA mutants in which negative regulatory modifications by PKA and Sch9 are prevented, promote the nuclear accumulation of Maf1 and its interaction with the polymerase, but in the absence of additional changes transcription is not efficiently repressed (35, 52). Similarly, altering the regulatory capacity of Rpc53 at three critical phosphosites has no effect on transcription in the absence of an appropriately sensitized strain background (11). Recent experiments in our laboratory have revealed another example of this phenotypic robustness involving the phosphoregulation of Bdp1.[4] Four functionally important phosphosites have been identified in Bdp1 at serines 49, 164, 178, and 586. Three of these sites, Ser-49, -178, and -586, conform to a CK2 consensus motif and CK2 *in vitro* kinase assays have shown that 90% of the labeling occurs at serines 49 and/or 586. Importantly, Ser to Ala mutations at all four sites (Bdp1 4SA) in combination with a hypomorphic *MAF1-myc* allele created a strain in which pol III

[4] J. Lee, R. D. Moir, and I. M. Willis, RNA polymerase III and TFIIIB phosphoregulation in *Saccharomyces cerevisiae*, unpublished data.

transcription is hyper-repressible. This phenotype was not observed with the double mutants S164A/S178A or S49A/S586A.[4] Given the apparent sensitivity of the Bdp1 4SA strain but not the Bdp1 S164A/S178A strain to phosphorylation at CK2 sites, we employed the latter strain to assess the effect of Ckb1 phosphosite mutants on pol III hyper-repressibility. We reasoned that if Ckb1 phosphorylation has a negative effect on CK2 activity toward Bdp1, the S111E mutant of Ckb1 may phenocopy the hyper-repressibility of Bdp1 4SA upon rapamycin treatment.

Pol III activity in the tested strains was normalized to an untreated *BDP1-HA* wild-type strain containing a chromosomal wild-type *CKB1-HA* allele. Pre-tRNA^{Leu} synthesis in this strain was reduced to 36% after rapamycin treatment (Fig. 7, *B* and *C*) consistent with previous studies.[4] In the *BDP1-HA 4SA* strain, pre-tRNA^{Leu} synthesis was reduced ~50% during log-phase and demonstrated the hyper-repressible phenotype after rapamycin treatment (Fig. 7, *B* and *C*). Pre-tRNA^{Leu} synthesis in *BDP1-HA S164A/S178A* strains was also diminished in log-growth and ranged from 55 to 75% of the level seen in the control strain. This reduced level of synthesis was dependent on the *CKB1-HA* allele because it was not observed in an untagged *CKB1* strain background.[4] In the presence of *BDP1-HA S164A/S178A*, strains containing wild-type *CKB1-HA* or its S111A mutant exhibited comparable levels of repression by rapamycin. In contrast, the phosphomimetic *CKB1-HA S111E* strain was statistically less efficiently repressed (Fig. 7, *B* and *C*). Clearly, the combination of *CKB1-HA S111E* and *BDP1 S164A/S178A* did not recapitulate the hyper-repression seen in the *BDP1 4SA* strain (Fig. 7, *B* and *C*). Instead, the properties of the S111E mutant suggest an unexpected role for CK2 in attenuating the repression of pol III transcription under stress.

DISCUSSION

The identification of Kns1 and Mck1 as important regulators of ribosome and tRNA synthesis downstream of TORC1 led us to investigate roles for these kinases in other TORC1-regulated processes. In this work, we identified 220 gene deletions that increased the rapamycin sensitivity of the *kns1*Δ *mck1*Δ strain. Ninety-one of these gene deletions were not previously known to confer rapamycin sensitivity. Since the completion of the screen, nine of the genes in this group have been assigned a rapamycin-sensitive phenotype in other studies (see supplemental Table S1), thereby validating our strategy to uncover new TORC1 connectivity. Our results provide an enriched set of genes associated with TORC1-regulated biology and suggest new roles for Kns1 and Mck1 in regulating many processes including autophagy and vesicle-mediated transport.

The N terminus of human CK2β has previously been characterized as a site for CK2 autophosphorylation and has been implicated in regulating CK2β protein stability (39, 53). In yeast, multiple *in vitro* experiments have shown Ckb1 to be phosphorylated by the α subunits (43, 54). We show that Kns1 directly and differentially phosphorylates Ckb1 at Ser-111 under multiple stress conditions (Fig. 2*B*). Additionally, no significant phosphorylation was evident at sites other than Ser-111 in either the *kns1*Δ strain or in the *CKB1 S111A* strain. Thus, at least in yeast, CK2 autophosphorylation of the β subunits *in vivo* is negligible.

No crystal structure is available for the β subunits of *S. cerevisiae* CK2 but sequence alignment of regulatory subunits across species places Ser-111 adjacent to a conserved internal acidic loop, within a 30-amino acid insertion in yeast Ckb1 (14, 15, 55). The integrity of the acidic loop is important for the *in vitro* phosphorylation of human CK2 and for the activation of CK2 by polyamines (50). Thus, although the specific phosphorylation site in Ckb1 is not conserved in human CK2β, a shared mechanism may exist if the human β subunit is found to be phosphoregulated near the acidic loop. Positional (local) rather than precise conservation of phosphoregulatory sites have been noted previously for Maf1 (3) as well as for other proteins (56).

The phosphorylation of Ckb1 under all tested repressing conditions suggested a role for this modification in the regulation of CK2 function under stress. A possible mechanism proposed in a previous study involved the dissociation of CK2 catalytic subunits from the holoenzyme following DNA damage (20). However, coimmunoprecipitation of CK2 subunits did not support this hypothesis using either DNA damage or growth to high cell density as a perturbation. Differences between this study and previous findings may be explained in part by our use of native promoters and chromosomally tagged strains instead of a heterologous promoter driving expression of GST-tagged subunits expressed from high copy 2-μm plasmids (20). Glycerol gradient analysis also did not reveal changes in CK2 subunit distribution after tunicamycin treatment or in the Ckb1 S111E phosphosite mutant (Fig. 5, *A* and *B*). These data strengthen our conclusion that Ckb1 phosphorylation does not promote the dissociation of catalytic and regulatory subunits, and that CK2 subunit dissociation is not a general mechanism for the regulation of CK2 activity in *S. cerevisiae*.

Based on the contribution of CK2 to pol III transcription in yeast and other eukaryotes and its association with pol III genes in multiple species (19–22, 31, 49), we found that CK2 is differentially associated with tRNA genes under stress. Thus, differences in the frequency or lifetime of CK2 binding to tRNA gene loci may be an important mechanism for regulating pol III transcription. We noted that the dissociation of CK2 from pol III loci was not complete after rapamycin treatment (Fig. 6, *B* and *C*). This may indicate a role for CK2 in signal desensitization under repressing conditions or in preparing the system to restore transcription.

An analysis of Ckb1 phosphosite mutants by ChIP and Northern blotting revealed either no changes relative to wild-type Ckb1 or changes that were contrary to the simple model correlating Ckb1 phosphorylation with reduced CK2 occupancy of tRNA loci and repression of pol III transcription (*e.g.* Ckb1 S111E, Figs. 6*B* and 7*C*). As seen in other studies of the pol III system (11, 35),[4] phosphosite mutants of individual components in otherwise wild-type strains have little or no effect on transcription. This robustness of the system against perturbations can be weakened by combining functionally appropriate changes in multiple proteins. However. our approach to unmask the contributions of Ckb1 phosphorylation using Bdp1 phosphosite mutants was not successful. The combination of Bdp1 S164A/S178A, which retains CK2 sites at Ser-49 and Ser-

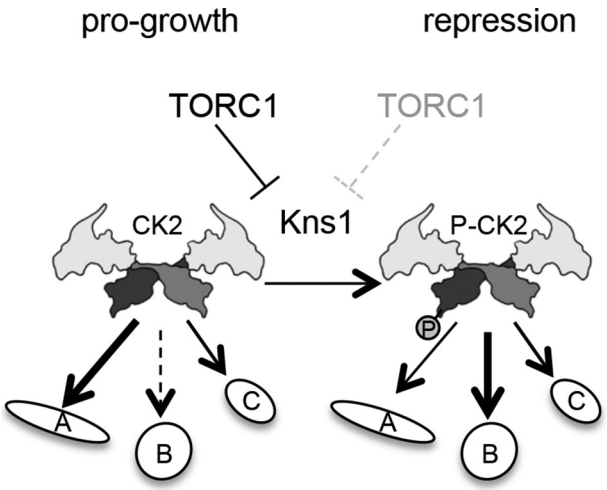

FIGURE 8. **Model of CK2 regulation via Ckb1 phosphorylation.** In optimal growth conditions, TORC1 inhibits Kns1 protein expression and CK2 phosphorylates various targets (including Bdp1) and promotes pol III transcription. With nutrient limitation, DNA damage, or other stresses, TORC1 activity is inhibited resulting in induction of Kns1 expression and its activation and enrichment in the nucleus. This results in Kns1 phosphorylation of Ckb1 and repression of pol III transcription. Phosphorylation of Ckb1 is proposed to affect the affinity of CK2 for substrate proteins in many processes. Changes in affinity may be deactivating (*e.g.* target *A*), activating (*e.g.* target *B*), or may not affect the interaction between CK2 and some target (*e.g.* target *C*). *Line thickness* indicates the relative strength of association of CK2 to its targets.

586 with Ckb1 S111E was clearly not equivalent to Bdp1 4SA with respect to the hyper-repressible response to rapamycin. Additional genetic buffering effects are presumably at work. These could include (i) the presence of free catalytic CK2 subunits that can phosphorylate Bdp1 even though the association of the CK2 holoenzyme with pol III loci is reduced, and (ii) rapid re-phosphorylation of Bdp1 after its initial rapamycin-induced dephosphorylation. In any event, the phosphorylation of Bdp1 at Ser-49 and/or Ser-586 is sufficient to limit repression of transcription compared with that seen for Bdp1 4SA.

Although the functional consequences of Ckb1 phosphorylation for pol III transcription remain an open question, the high stoichiometry of the modification in nutritionally starved or tunicamycin-treated cells indicates that Kns1 has the potential to alter the function of almost half the estimated 8000 molecules of CK2 holoenzyme per cell (57). Several factors suggest an important *in vivo* role for the phosphorylation of Ckb1. First, Kns1 is differentially expressed and translocates to the nucleus under stress (11), where CK2 is most abundant (44). Second, Kns1 plays a key role in the phosphorylation of Rpc53 under conditions identical to those leading to Ckb1 phosphorylation (11). Third, the Ckb1 phosphosite, Ser-111, is part of an extended solvent-exposed acidic loop that binds polyamines, which stimulate CK2 activity (50). Fourth, the acidic loop of the β subunit is a likely docking site for positively charged target proteins (14). Therefore, it is an attractive hypothesis that the phosphorylation of Ckb1 at Ser-111 may alter the affinity of the holoenzyme for its substrates (Fig. 8). The increase in negative charge near the acidic loop may promote the association of proteins with exposed basic patches, reduce the affinity for negatively charged proteins, or have no effect on the association of CK2 to a third set of its substrates. Accordingly, the biological significance of Ckb1 phosphoregulation may be uncovered by studies of CK2 associations in response to stress. Potential phosphoregulated CK2 interactions may include the translation initiation factor eIF3 (58), the Paf1 transcription elongation complex (59), and the heat-shock factor Hsf1 (60). A more comprehensive analysis of these and other *in vivo* CK2 targets in strains containing Ckb1 phosphosite mutants may provide evidence supporting the proposed role for Ckb1 phosphorylation in the regulation of CK2 binding to its substrates.

In summary, these experiments uncovered a new connection in *S. cerevisiae* between the master kinase TORC1, its downstream effector Kns1, and the differential phosphorylation of a subunit of protein kinase CK2, implicating the existence of additional regulatory mechanisms that govern the contributions of CK2 to ribosome and tRNA biogenesis and potentially other fundamental cellular processes.

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